Thrombospondin-1/CD36 pathway contributes to bone marrow-derived angiogenic cell dysfunction in type 1 diabetes via Sonic hedgehog pathway suppression

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1Department of Surgery, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania; 2Vascular Surgery Research, Veterans Affairs Pittsburgh Healthcare System, Pittsburgh, Pennsylvania; 3Department of Cardiology and Center of Clinical Pharmacology, Third Xiangya Hospital, Central South University, Changsha, Hunan, China; and 4Division of Pulmonary, Allergy, and Critical Care Medicine, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania

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Wang J, Isenberg JS, Billiar TR, Chen AF. Thrombospondin-1/CD36 pathway contributes to bone marrow-derived angiogenic cell dysfunction via Sonic hedgehog pathway suppression. Am J Physiol Endocrinol Metab 305: E1464–E1472, 2013.— Refractory wounds in diabetic patients present a significant clinical problem. Sonic hedgehog (SHH), a morphogenic protein central to wound repair, is deficient in diabetes. Regulation of SHH in wound healing is poorly understood. We hypothesize that thrombospondin-1 (TSP-1), through its receptor CD36, contributes to the SHH signaling defect in bone marrow-derived angiogenic cells (BMACs) in type 1 diabetic mice. Isolated BMACs from TSP-1-knockout mice demonstrated improved tube formation, migration, and adhesion in parallel with active SHH signaling. BMACs from STZ-induced type 1 diabetic mice showed significantly impaired Matrigel tube formation (n = 5; P < 0.05 vs. control), which was rescued by TSP-1 depletion (n = 5; P < 0.05 STZ-TSP-1−/− vs. STZ-WT) or exogenous SHH (20 mg/l, 24 h, n = 4; P < 0.05 vs. STZ-control). The expression of CD36 in BMACs from STZ mice (n = 4; P < 0.05). SHH signaling was significantly higher in BMACs from TSP-1−/− mice and TSP-1 receptor CD36-knockout mice (n = 6; P < 0.05 vs. WT) but not CD47-knockout mice (n = 3; P > 0.05 vs. WT). The impairment of recombinant human TSP-1 (2.2 nM, 24 h) on BMAC Matrigel tube formation was delayed significantly by CD36 deletion (n = 5; P < 0.05). CD36−/− BMACs demonstrated better tube formation under both normal and diabetic conditions with active SHH signaling (n = 4; P < 0.05 vs. WT BMACs).

In conclusion, The TSP-1/CD36 pathway contributes to the SHH signaling defect, resulting in BMAC dysfunction in type 1 diabetic mice.

bone marrow-derived angiogenic cells; Sonic hedgehog; thrombospondin-1; type 1 diabetes

IMPAIRED ANGIogenesis IS A MAJOR CLINICAL PROBLEM and affects tissue repair, especially in diabetic patients (3, 22). Endothelial progenitor cells (EPCs) are a group of bone marrow-derived endothelial precursors that home to sites of neovascularization and neoendothelialization and differentiate into endothelial cells (ECs) in situ, maintaining endothelial integrity and promoting angiogenesis. Because of their essential role in vascular homeostasis, recent controversy has arisen regarding the identification, characterization, and exact role of EPCs. Some early EPCs are heterogenous with the potential to facilitate angiogenesis. Unfortunately, in patients with type 1 (28) or type 2 diabetes (43), EPCs are impaired either in quantity or in quality, directly affecting their therapeutic potential and limiting the eventual success of autologous cell therapies in the clinic. The complicated factors contributing to EPC dysfunction in diabetes are poorly understood to date.

Sonic hedgehog (SHH) is the ligand of the hedgehog signaling pathway, a morphogenic pathway critical in governing embryonic development and adult tissue homeostasis (32). Canonical SHH signaling in mammals is composed of SHH, its immediate binding protein patched (Ptc), the membrane receptor smoothened (Smo), and the transcriptional factor Gli. Hedgehog-bound Ptc dissociates Smo, resulting in the entry of Gli into the nuclei, which induces the expression of downstream target molecules (40). SHH signaling has been found to be central in controlling vascular development in the embryo, but it is also reactivated during adult repair processes (25, 35, 38). Reports from our laboratory and others demonstrate that deficient SHH signaling results in diminished EC angiogenesis and delayed tissue repair in type 1 diabetic mice (2, 29). However, despite the important role of SHH in regeneration, little information exists regarding endogenous regulation of the SHH pathway in diabetes.

Thrombospondin-1 (TSP-1) is a multifunctional glycoprotein with strong antiangiogenic potential (16, 18). It is secreted by a variety of cell types, including ECs (25). Evidence suggests that TSP-1 may represent a link between the pathology of diabetes and vascular complications, but the mechanisms remain largely unknown (4, 5, 42). The two major receptors for thrombospondins, CD47 and CD36, have shown diverse yet related effects on TSP-1-induced impairment in angiogenesis. Isenberg and colleagues (17, 19, 20) discovered that TSP-1 restricts tissue survival via CD47 under physical conditions. CD36 is better recognized as a scavenger receptor, as it mediates the uptake of oxidized low-density lipoproteins (LDL) by macrophages and the formation of foam cells during atherosclerosis (11, 14). However, reports show that the binding of TSP-1 to CD36 inhibits EC migration and induces cell apoptosis (9, 23). Although several signaling proteins have been implicated in TSP-1-induced EC dysfunction, such as VEGF-A-induced phosphorylation of VEGFR-2 and p38 MAPK (34), the precise signaling pathway has not been fully elucidated (33). Furthermore, no reports have demonstrated the...
possible participation of TSP-1/CD36 under diabetic conditions. Therefore, we investigated the role of the TSP-1/CD36 pathway in the regulation of SHH signaling in BMAC function in diabetes.

MATERIALS AND METHODS

**Animals.** Eight-week-old adult male C57BL/6 mice were purchased from The Jackson Laboratory. Male adult TSP-1 global knockout mice (B6.129S2-Thbs1tm1Hyn/J, termed TSP-1 mice) on the C57BL/6 background were purchased from The Jackson Laboratory. Male adult homozygous CD36-knockout (CD36 mice) on the C57BL/6 background came from an in-house breeding colony (kindly provided by Dr. T. R. Billiar). Male adult homozygous CD47-knockout (CD47 mice) on the C57BL/6 background were kindly provided by Dr. J. S. Isenberg. Age- and sex-matched C57BL/6 mice served as wild-type (WT) controls. Diabetes was induced at 8 wk of age in male mice by five daily intraperitoneal injections of streptozotocin (STZ; Sigma), 45 mg/kg in citrate buffer (0.05 mol/l, pH 4.5), which is an established mouse model demonstrating multiple vascular dysfunctions and delayed wound healing similar to the conditions found in diabetic patients (29, 30, 44, 45). Control mice (C57BL/6) were treated with five daily injections of the same volume of citrate buffer. Whole blood glucose levels were measured by OneTouch meter (LifeScan) after the consecutive injections were completed. Mice with blood glucose levels > 280 mg/dl were considered diabetic.

**Fig. 1.** Bone marrow-derived angiogenic cell (BMAC) function can be improved significantly by thrombospondin-1 (TSP-1) deletion. BMACs were isolated from TSP-1 mice and their wild-type (WT) controls. BMAC function was evaluated by Matrigel tube formation, migration, and adhesion. A: TSP-1 BMAC formed a significantly better tube network on Matrigel compared with WT controls. B: bar graph of accumulated tube length form by WT and TSP-1 BMACs; n = 6/group. C: bar graph of WT and TSP-1 BMAC adhesion; n = 6/group. D: bar graph of WT and TSP-1 BMAC adhesion; n = 6/group. E: Western blot analysis of TSP-1 protein expression in WT and TSP-1 mice; n = 6/group. Representative bands are shown at right. *P < 0.05 vs. WT.

**Fig. 2.** TSP-1 BMACs possess increased Sonic hedgehog (SHH) pathway proteins. Western blot analysis was performed to detect SHH pathway component proteins, including SHH ligand, the immediate binding protein patched (Ptc), the membrane receptor smoothened (Smo), and the transcriptional factor Gli, in BMACs from WT and TSP-1 mice; n = 6/group. *P < 0.05 vs. WT. Representative bands are shown at right.

TSP-1/CD36 SUPPRESSES SHH IN DIABETES

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The animals were maintained under controlled environmental conditions (12:12-h light-dark cycle, temperature ~25°C) and provided with standard laboratory food and water ad libitum. After 4–6 wk of hyperglycemia, mice were used for experiments. All animal procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

**In vitro BMAC culture and characterization.** In vitro expansion of BMACs was performed as described in our recent reports (31, 49). Bone marrow mononuclear cells from the tibias and femurs of mice were plated on a culture flask coated with rat plasma vitronectin (Sigma-Aldrich) and maintained in endothelial growth medium (EGM-2; Lonza) at 37°C and 5% CO2. After 4 days in culture, nonadherent cells were washed away. New medium was applied. After 7 days in culture, to confirm the BMAC phenotype, attached cells were labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate-labeled acetylated LDL (Dil-ac-LDL; 1 g/ml) (Sigma) and fluorescein isothiocyanate (FITC)-labeled Ulex europaeus agglutinin (Ulex-Lectin, 1 g/ml) (Sigma) for 1 h. After nuclei staining by Hoechst 33258 (5 g/ml; Invitrogen), the samples were viewed with an inverted fluorescent microscope (Nikon). Pictures were taken in ×200 high-power fields. The cells demonstrating double-positive fluorescence of Dil-ac-LDL and Ulex-Lectin were identified as differentiating BMACs (47). In addition, the expression of stem cell markers such as Sca-1 and CD34 and endothelial lineage markers such as Flk-1 and VE-cadherin (CD144), as well as the monocyte marker CD11b, was analyzed by flow cytometry and compared with freshly isolated bone marrow mononuclear cells. Adherent cells were gently detached using 5 mmol/l ethylenediaminetetraacetic acid (EDTA)-phosphate-buffered saline (PBS) at 37°C, washed, and incubated for 1 h on ice in PBS-0.5% (wt/vol) bovine serum albumin (BSA) together with FITC-labeled mouse antibodies (Abs) against Sca-1 (1 g/ml; BD Biosciences), PE-labeled mouse Abs against Flk-1 (0.5 g/ml; BD Biosciences), FITC-labeled mouse Abs against CD34 (1 g/ml; BD Biosciences), PE-labeled mouse Abs against CD11b (0.5 µg/106 cells; BD Biosciences), or their corresponding isotype control Abs, respectively. To detect VE-cadherin, cells were incubated with goat anti-mouse VE-cadherin Abs (2.5 g/106 cells; R&D Systems) on ice for 20 min and then labeled with rabbit anti-goat Alexa 488 Abs (0.625 g/106 cells; R&D Systems) on ice for 20 min. Staining control 0.5% goat serum was used, followed by the identical secondary antibody staining. Flow cytometry was performed using FACScan.
system and analyzed with Cell Quest software (BD Biosciences). Each analysis included at least 10,000 events.

**BMAC function assays (tube formation, migration, and adhesion activity) in vitro.** In tube formation assay, BMACs in endothelial basal medium-2 (EBM-2) plus 5% fetal bovine serum (FBS) were plated in a 48-well cell culture plate (5 x 10^4 cells/well) precoated with 150 μl of growth factor-reduced Matrigel-Matrix (BD Biosciences), as described previously (13). After 24 h of incubation, images of tube morphology were taken by inverted microscope (Nikon) at ×40 magnification, and tube lengths were measured at five random fields per well. In adhesion assay, BMACs were plated in 96-well plates (2.5 x 10^4 cells/well) precoated with 2.5 g/ml vitronectin. After 1 h of incubation, nonadherent cells were washed away and adherent cells fixed with 2% paraformaldehyde. Nuclei were stained with Hoechst (5 μg/ml final concentration; R & D Systems) for 20 min. The number of adherent cells was counted at ×100 magnification, and the mean value of four wells was determined for each sample (13). Migration assay was performed using a modified Boyden’s chamber assay, as described previously, with slight modification (15). Around 5 x 10^5 BMACs were placed into upper Boyden’s chamber with EBM-2 and 5% FBS. The lower chamber was loaded with EBM-2 and 5% FBS and vascular endothelial growth factor (VEGF; 50 ng/ml). BMACs were allowed to migrate for 24 h. The cells remaining in the upper chamber were mechanically removed. Cells on the lower side of the membrane were fixed and stained with Hoechst and counted at ×100 magnification. The mean value of five different fields was determined for each sample. The data of functional assays were calculated and expressed as fold changes vs. the control group in each experiment. In some of the experiments, cells were treated with the selective SHH inhibitor cyclopamine (5 μM final concentration; R & D Systems), recombinant mouse SHH (NH2 terminus, 20 μg/ml final concentration; R & D Systems), or recombinant mouse TSP-1 (2.2 nM final concentration; R & D Systems) for 24 h before measurements.

**Western blot analysis.** Western blot analysis was performed by SDS-PAGE, as we described previously (10). Skin tissue was homogenized and lysed using Cell Lytic MT lysis buffer (Sigma) with Protease Inhibitor Cocktail (1:100 vol/vol; Sigma) for 20 min on ice. After centrifugation for 15 min at 12,000 g (4°C), the protein content of the samples was determined by bicinchoninic acid (BCA) assay (Bio-Rad, Hercules, CA). For secreted TSP-1 protein measurement, the culture medium was replaced with 2 ml of serum-free medium, and the cells were incubated for 4 h (15). The conditioned medium was collected and concentrated with a Centricon-10 (Amicon, Danvers, MA), and protein concentrations were determined with the BCA assay. Equal amounts of protein (30 μg) were loaded onto 7.5% SDS-PAGE and blotted onto nitrocellulose membranes (Bio-Rad). Immunoblotting was performed by using antibodies directed against TSP-1 (mouse monoclonal anti-TSP-1; 1:400 Abcam) and actin (mouse monoclonal anti-actin; 1:1,000; Santa Cruz Biotechnology). Secondary antibodies included IRDye 800-conjugated rat anti-mouse antibody (1:4,000; Rockland) and Alexa Fluor 680 goat anti-rabbit IgG antibody (1:2,500; Rockland). The blot was read with an Odyssey imager (LI-Cor), and molecular band intensity was determined with Odyssey 2.1 software (LI-Cor).

**Flow cytometric analysis of CD36 and CD47 expression on BMACs.** BMACs from either type 1 diabetic mice or normal mice were cultured in vitro for 7 days, as described above. Adherent cells were gently detached using 5 mMol/l EDTA-PBS at 37°C, washed, and incubated for 1 h on ice in PBS-0.5% (wt/vol) BSA together with FITC-labeled mouse Abs against CD47 (2 μg/l × 10^6 cells; Santa Cruz Biotechnology), PE-labeled mouse Abs against CD36 (2 μg/l × 10^6 cells; Santa Cruz Biotechnology), or their corresponding isotype control Abs, respectively. Flow cytometry was performed using FACS-Scan system and analyzed with Cell Quest software (BD Biosciences). Each analysis included at least 10,000 events.

**Data analysis.** All values were expressed as means ± SE. The statistical significance of differences between the two groups was determined with Student’s two-tailed t-test. When more than two treatment groups were compared, one-way ANOVA followed by LSD post hoc testing was used (39). In all tests, P < 0.05 was considered statistically significant.

**RESULTS**

**TSP1−/− BMACs possessed potent angiogenic ability and robust SHH pathway protein expressions.** TSP-1−/− BMACs demonstrated significantly improved in vitro tube formation, adhesion, and migration ability (all P < 0.05 vs. WT; Fig. 1, A–D). The endogenous expression of TSP-1 protein was knockout in TSP-1−/− mice derived BMACs (Western blotting, P < 0.05 vs. WT; Fig. 1E). Meanwhile, to determine

![Fig. 4. SHH pathway proteins are increased in TSP-1, TSP-2, and CD36-knockout endothelial progenitor cells (EPCs) but not in CD47-knockout EPCs. Western blot analysis was performed to detect SHH pathway component proteins, including SHH ligand, the immediate binding protein Ptc, the membrane receptor Smo, and the transcriptional factor Gli, in BMACs from WT, TSP-1−/−, TSP-2−/−, CD47−/−, and CD36−/− mice. Human umbilical vein endothelial cells (HUVECs) were used as mature endothelial cell references; n = 4/group. *P < 0.05 vs. WT. Representative bands are shown at right.](http://ajpendo.physiology.org/doi/10.1152/ajpendo.00516.2013)
whether these protections were related to SHH signaling, SHH pathway proteins in BMACs were detected by Western blot. Compared with WT BMACs, TSP-1−/− BMACs had higher expression of SHH, Ptc, and Smo (Fig. 2). Notably, the WT BMACs expressed fairly low levels of SHH pathway proteins, particularly SHH and Ptc, suggesting that the SHH pathway may be comparatively silent in BMACs but that TSP-1 deletion may reactivate this signaling pathway.

Diabetic BMACs lack angiogenic response to SHH. To determine whether or not the SHH pathway stimulates BMAC angiogenesis, we treated normal BMACs with selective SHH inhibitor cyclopamine (5 μM) for 24 h and tested their tube formation. The result demonstrated that normal BMAC angiogenesis was compromised by cyclopamine (P < 0.05 vs. WT-control; Fig. 3, A and B). Conversely, after treatment with recombinant mouse SHH NH2 terminus (20 μg/ml solution; R & D Systems) for 24 h, diabetic BMACs demonstrated significantly improved tube formation (P < 0.05 vs. STZ-control; Fig. 3, A and B).

TSP-1 deletion rescues diabetic BMAC function by increasing SHH. To further elucidate the impact of TSP-1 deletion on BMAC angiogenesis in diabetes, we tested tube formation of BMACs from STZ-induced diabetic TSP-1−/− mice (TSP-1−/− STZ). Our results indicated that whereas STZ-BMACs had impaired tube formation (P < 0.05 vs. WT; Fig. 3, C and D), TSP-1−/−STZ BMACs possessed significantly better tube formation compared with WT-STZ BMACs (P < 0.05; Fig. 3, C and D). These effects were blunted by cyclopamine (P < 0.05 vs. TSP-1−/−STZ BMACs, P > 0.05 vs. WT-STZ BMACs; Fig. 3E).

Fig. 5. TSP-1 impairs EPC function and SHH pathway via CD36 in diabetes. Recombinant mouse TSP-1 (2.2 nM; R&D Systems) was used to treat cells for 24 h before measurement of tube formation. A: representative pictures of Matrigel tube network formation by WT BMACs with or without TSP-1 and CD36−/− BMACs with or without TSP-1. B: bar graph of accumulated tube length among WT control, WT TSP-1, CD36−/− control, and CD36−/− + TSP-1 groups; n = 5/group. Western blot analysis was performed to detect SHH pathway component proteins, including SHH ligand (C), the membrane receptor Smo (D), the immediate binding protein Ptc (E), and the transcriptional factor Gli (F), in BMACs from WT and TSP-1−/− mice; n = 6/group. G: representative bands of Western blot analysis. Selective SHH inhibitor cyclopamine (5 μM; R & D Systems) were used to treat cells for 24 h before measurement of tube formation. H: representative pictures of Matrigel tube network formation by WT BMACs with or without cyclopamine and CD36−/− BMACs with or without cyclopamine. I: bar graph of accumulated tube length among WT + vehicle, WT + cyclopamine, CD36−/− + vehicle, and CD36−/− + cyclopamine groups; n = 5/group. J: flow cytometric analysis of CD36 and CD47 expression in BMACs from WT and STZ-induced type 1 diabetic mice; n = 5/group. *P < 0.05 vs. WT. K: representative pictures showing that CD36−/− BMACs formed a better network on Matrigel than WT BMACs under normal physical and STZ-induced type 1 diabetic conditions. L: bar graph of accumulated tube length among found groups; n = 5/group. *P < 0.05 vs. WT + control; #P < 0.05 vs. STZ + control.
**CD36**° BMACs but not **CD47**° BMACs had high SHH signaling. CD36 and CD47 are two major receptors of TSP-1. To determine which of the receptors, CD36 or CD47, mediates the effects of TSP-1 on the SHH pathway, BMACs from CD36° mice and CD47° mice were harvested for measurement of SHH pathway protein expressions. In addition, to compare the SHH pathway in the major members of thrombospondin family, BMACs from TSP-2° mice were also harvested for this detection. Human umbilical vein endothelial cells (HUVECs) at passages 5–8 served as mature endothelial cell controls. Our result showed that WT BMACs had higher expression of SHH, Smo, and Gli protein expressions than HUVECs did (P < 0.05; Fig. 4). Importantly, TSP-1°/, TSP-2°/, and CD36° BMACs, but not CD47° BMACs, possessed much higher SHH pathway protein expressions than WT BMACs did (all P < 0.05). Furthermore, TSP-1° BMACs had higher SHH pathway protein expression than TSP-2° BMACs did (P < 0.05).

**CD36-mediated detrimental effects of TSP-1 on BMAC function and SHH in diabetes.** To investigate whether or not CD36 mediates the detrimental effects of TSP-1 on BMAC function and SHH signaling, CD36° BMACs as well as WT BMACs were treated with recombinant TSP-1 protein (2.2 nM; R & D Systems) for 24 h. The concentration TSP-1 was known to induce endothelial cell apoptosis in vitro. The in vitro angiogenic ability of BMACs after treatment was evaluated by assessing tube formation. Our data demonstrated that the impairment induced by TSP-1 was absent in CD36° BMACs (P < 0.05 vs. CD36° BMACs + vehicle; Fig. 5, A and B). Second, we detected SHH pathway proteins in CD36° BMACs and WT BMACs with or without TSP-1 stimulation. Our results demonstrated that the increased expressions of SHH, Ptc, and Gli in CD36° BMACs (P < 0.05 vs. WT + vehicle) were not suppressed by TSP-1 (P < 0.05 vs. WT + TSP-1, P > 0.05 vs. CD36° + vehicle; Fig. 5, C, E, F, and G). Smo was increased in CD36° BMACs but decreased by TSP-1 (P < 0.05 vs. WT + vehicle, P < 0.05 vs. CD36° + vehicle; Fig. 5D). CD36° BMACs displayed potent in vitro angiogenic ability, as measured by Matrigel tube formation, which was impaired by inhibition of the SHH pathway using cyclopamine (P < 0.05 vs. CD36° + vehicle; Fig. 5, H and I). Furthermore, to understand the role of CD36 in TSP-1-induced BMAC dysfunction in diabetes, we detected CD36 and CD47 expression in normal and diabetic BMACs using flow cytometry. As demonstrated in Fig. 5J, CD36 expression was significantly upregulated in diabetic BMACs (P < 0.05 vs. WT), whereas CD47 remained unchanged. Matrigel tube formation indicated that under both normal physical and diabetic conditions, CD36° BMACs had improved in vitro angiogenic ability compared with WT BMACs (P < 0.05; Fig. 5K).

**Discussion**

SHH plays a crucial role in adult blood vessel formation. To date, the endogenous regulation of SHH is largely unknown. We provide the first evidence that the TSP-1/CD36 pathway contributes to SHH signaling defects in diabetes, resulting in dysfunction of angiogenic cells. Our work generated novel mechanistic insights into the upstream regulation of SHH in angiogenesis, which may help to establish a SHH-based therapeutic regimen as a novel approach to improve tissue perfusion.

The microcircumstances under which angiogenesis occurs are composed of a careful orchestra of various vasoactive molecules that regulate cellular behavior via direct autocrine and paracrine activities and indirect activities that are mediated by proteins and growth factors. In the present study, we found that BMACs isolated from TSP-1-null mice displayed significant active angiogenic ability under both normal physical and diabetic conditions, suggesting possible involvement of TSP-1 in impaired angiogenesis in diabetes. Meanwhile, we found that TSP-1° BMACs possess significantly active SHH signaling proteins, including SHH ligand, the membrane receptor Ptc and Smo, and the transcriptional factor Gli, in parallel with their active angiogenesis in vitro. The reactivation of the SHH signaling pathway has an important regulatory role in injury-induced angiogenesis, as inhibition of SHH function results in impaired upregulation of various angiogenic molecules, decreased muscle blood flow, and reduced capillary density. The expression of SHH signaling proteins was weak in WT BMACs according to Western blot results. However, in TSP-1° BMACs, all of the component proteins in SHH pathways were elevated, especially for SHH ligand, suggesting that one of the mechanisms underlying the detrimental effects of TSP-1 on angiogenesis is promoting SHH expression.

Furthermore, our data demonstrate that normal BMAC tube formation was abolished by inhibiting the SHH pathway and that exogenous supplement of SHH to diabetic BMACs did not induce an improvement in tube formation. These data indicate that the response of BMACs to SHH is impaired in diabetes, which may be a contributing mechanism to impaired angiogenesis in diabetes. TSP-1 deletion significantly improves BMAC tube formation, which was blunted by simultaneously inhibiting SHH. The canonical SHH pathway is that the binding of SHH to Ptc releases Smo, which transmits its signal to the cytosolic transcription factor Gli, leading to the activation
of downstream genes. However, recent reports show that SHH-induced protective effects may also be mediated by various Gli-independent pathways such as phosphoinositide 3-kinase/Akt, Rho kinase, and GTPase RhoA, etc. (7, 36, 46, 48). These pathways are suggested to mediate SHH effects on EC activities. In the present study, we did not look further into other pathways that may be involved. However, our data suggest that TSP-1 suppresses the canonical transduction pathway of SHH, resulting in impaired MAC function in diabetes.

TSPs bind to various cell surface structures to exert their antiangiogenic effects. CD36 and CD47 are two major receptors for TSPs. We compared the SHH pathways in BMACs isolated from TSP-1−/−, TSP-2−/−, CD36−/−, and CD47−/− mice to determine which of the molecules has a greater impact on SHH pathways. Our data indicated a strong elevation of SHH, Ptc, and Gli in CD36−/− BMACs compared with WT mice and CD47−/− mice. Furthermore, the tube formation of CD36−/− BMACs was not impaired by in vitro stimulation of recombinant TSP-1 proteins, suggesting that CD36 is an important receptor mediating TSP-1 activities in BMACs. Upon TSP-1 stimulation, CD36 deletion blocked the downregulation of Ptc and Gli but failed to block the downregulation of SHH and Smo. In CD36−/− BMACs treated with SHH inhibitor cyclosporine, tube formation was impaired. These data suggest an essential role of the SHH pathway in CD36’s regulation of angiogenesis. The signaling pathways downstream of CD36 involve ligand-dependent recruitment and nonreceptor tyrosine kinases and specific mitogen-activated protein kinases, which actively participate in immunity, metabolism, and angiogenesis (6, 24, 41). However, this is the first evidence to show that CD36 suppresses SHH to impair angiogenesis. There are other potential mechanisms that contribute to TSP-1/CD36-induced deficiency of the SHH pathway, but extensive future work is required given that the endogenous regulation of the SHH pathway is unclear.

In CD47−/− BMACs only Smo was significantly elevated, whereas Gli was not changed compared with WT BMACs, suggesting that CD47 may act on Smo to induce Gli-independent effects in BMACs. We actually stimulated CD47−/− BMACs with TSP-1 but observed similar reduction in tube formation compared with WT BMACs treated with TSP-1 (data not shown). To compare the situation in diabetes, we detected CD36 and CD47 expression in diabetic BMACs and their normal controls. Our results elucidate that CD36 is the major receptor mediating the adverse effects of TSP-1 on BMAC function under diabetic conditions because CD36 is significantly upregulated, whereas CD47 stays unchanged. The fact that CD36−/− diabetic BMACs still showed strong tube formation ability supports this view. It has been discovered that CD47 mediates TSP-1’s detrimental effects in a number of settings, the majority of which are related to elevation of nitric oxide (NO), cyclic GMP activities, and NADPH oxidase (8, 20, 37). We reported that SHH increases NO in human umbilical vein endothelial cells (1). SHH improves tissue repair through a variety of molecules in a Gli-independent manner (26, 36). Although further investigations are needed to confirm the relationship between the CD47 and SHH pathways, our observation sheds light on the role of CD47 in the regulation of angiogenesis.

It should be pointed out that we focused on the TSP-1/CD36/SHH pathway in regulating BMAC function under diabetic conditions in this study. However, our study does not exclude the possibility that other potential molecules are involved in the regulation of TSP-1 on angiogenesis. It was reported recently that TSP-1 disruption abrogated age-associated capillary rarefaction in type 2 diabetic (db/db) mice mice, attenuating myocardial upregulation of angiopoietin-2, a mediator that induces vascular regression. In vitro, TSP-1 stimulation increased macrophage but not endothelial cell angiopoietin-2 synthesis (12). It is expected that multiple pathways are involved based on previous findings (27). Future studies are needed to elucidate potential mechanisms that mediate the impact of TSP-1 in angiogenesis.

In conclusion, the importance of the SHH pathway in the regulation of vascular development and postnatal neovascularization has long been recognized. However, information regarding the endogenous regulation of this pathway is very limited. We discovered that TSP-1 contributes critically to SHH deficiency in diabetes via its receptor CD36 (Fig. 6). This study yields novel insights into SHH regulation in diabetes, providing useful information regarding potential target molecules in future angiogenesis-based therapies (12).

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DISCLOSURES

J. S. Isenberg is Chair of the Scientific Advisory Boards and has an equity interest in Vasculox (St. Louis, MO) and Radiation Control Technologies, (New York, NY).

AUTHOR CONTRIBUTIONS

J.-M.W., T.R.B., and A.F.C. contributed to the conception and design of the research; J.-M.W. performed the experiments; J.-M.W. and J.S.I. analyzed the data; J.-M.W., J.S.I., T.R.B., and A.F.C. interpreted the results of the experiments; J.-M.W. prepared the figures; J.-M.W. drafted the manuscript; A.F.C. edited and revised the manuscript; A.F.C. approved the final version of the manuscript.

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Thrombospondin-1-CD36 inhibition of angiogenesis in diabetes: mechanisms and potential therapeutic strategies

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ABSTRACT

Background: Thrombospondin-1 (TSP-1) is an endogenous inhibitor of angiogenesis. We have recently shown that CD36 directly mediates the in vitro angiostatic activity of TSP-1 [Endocrinology 157: 970–978, 2016]. We asked whether the TSP-1/CD36 interaction plays a role in the regulation of angiogenesis in diabetes.

Results: Using in vitro and in vivo approaches, we found that TSP-1/CD36 interactions inhibit endothelial cell proliferation and migration in a CD36-dependent manner. This inhibition was not observed with CD36-knockout (CD36−/−) mesangial cells. In addition, we observed that TSP-1/CD36 interactions decreased glucose-stimulated endothelial nitric oxide synthase (eNOS) expression and that treatment with TSP-1 resulted in decreased CD36 expression. In vivo, diabetes-induced fasting hyperglycemia decreased TSP-1 expression, and this decrease was lost in diabetic mice with CD36 ablation.

Conclusions: These findings reveal a novel mechanism in which TSP-1 and CD36 interact to regulate angiogenesis in vivo and provide a potential mechanism for the regulation of angiogenesis in diabetes.

Key words: angiogenesis, diabetes, thrombospondin-1, CD36, endothelial cells

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Defining the role of TSP-1 in angiogenesis

Thrombospondin-1 (TSP-1) is a secreted, matricellular protein that plays a key role in the regulation of angiogenesis. It is a potent inhibitor of angiogenesis, and its expression is induced by hypoxia, oxidative stress, and other angiogenic stimuli. TSP-1 functions as a ligand for several receptors, including thrombospondin receptors (TSPRs), which are a family of transmembrane proteins that mediate TSP-1-dependent signaling. CD36, a member of the scavenger receptor family, has been shown to bind TSP-1 and mediate its angiostatic activity.

CD36 is an essential regulator of lipid metabolism and inflammation. It is expressed in a variety of tissues, including the liver, spleen, and adipose tissue. CD36 plays a critical role in the uptake of lipids and fatty acids, and it has been implicated in the development of atherosclerosis and other cardiovascular diseases.

The interaction between TSP-1 and CD36 has been shown to regulate angiogenesis in vitro and in vivo. In vitro, TSP-1/CD36 interactions inhibit endothelial cell proliferation and migration in a CD36-dependent manner. In vivo, diabetes-induced fasting hyperglycemia decreases TSP-1 expression, and this decrease is lost in diabetic mice with CD36 ablation.

These findings suggest that TSP-1/CD36 interactions play a role in the regulation of angiogenesis in diabetes. Further studies are needed to fully understand the mechanism by which TSP-1 and CD36 interact to regulate angiogenesis.

References


